In Situ Detection of Gene Transfer and Microbial Activity in a Model Biofilm Engaged in Degradation of Benzyl Alcohol

Bjarke Bak Christensen, Claus Sternberg, Jens Bo Andersen, Søren Molin.

Department of Microbiology, Technical University of Denmark, Building 301, DK-2800 Lyngby, Denmark

ABSTRACT

Establishment and transfer of the TOL-plasmid (pWWO) were studied in a model biofilm consisting of three different bacterial species engaged in benzyl alcohol degradation. Combining quantitative determination of donor, recipient and transconjugants with *in situ* monitoring of single cells through zygotic interaction of Gfp fluorescence provided hitherto unknown details about spread of the TOL-plasmid in a biofilm. Kinetic studies showed that the dominant mode for plasmid spread was vertical transfer of the transconjugants (growth) caused by the selective advantage provided by the TOL-plasmid. However, the microscopic analysis showed that, despite their selective advantage, the transconjugants established only on the surface of the recipient microcolonies. Transfer of the plasmid into the middle of the microcolonies was never observed. It is known that only cells with high activity transfer the TOL-plasmid efficiently. Therefore, in a separate biofilm experiment the recipient strain was replaced with a new monitor strain, carrying a growth phase regulated ribosomal RNA promoter *rrnB* P1 fused to an unstable variant of the *gfp* gene. This allowed for monitoring changes in the growth physiology of the organisms in the biofilm. The patterns observed for growth activity in the microcolonies turned out to be strikingly similar to the patterns observed for the establishment of transconjugants. This correlation between activity and transfer may suggest that there is a nutrient diffusion gradient through a micro-colony allowing growth of only the surface cell, and that plasmid transfer only occurs between these actively growing cells.

Introduction

Since Lederberg discovered in 1949 that genes can be horizontally transferred (the conjugative process) between bacteria, numerous studies have been performed to understand the function and nature of this process. Much information has been obtained by disintegrating microbial communities, followed by selective plating, but other methods, such as nucleic acid probes targeting specific DNA-regions, have frequently been used to trace specific plasmids.

Due to their high cell densities, biofilms are often considered as an ideal environment for plasmid transfer. However, the high cell densities may limit diffusion of nutrients into the inner layers of the biofilm, resulting in a heterogeneous distribution of the bacterial activity in the biofilm. For instance, transfer of the TOL-plasmid has been shown to depend on the growth activity of the bacteria [6], which may indicate the existence of a correlation between spread of plasmids and the physiological state of the organisms in the community. To determine whether such a relationship exists, traditional enumeration

Microbial Biosystems: New Frontiers
Proceedings of the 8th International Symposium on Microbial Ecology
Bell CR, Brylinsky M, Johnson-Green P (ed)
Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 1999.

techniques have to be combined with other techniques that allow for *in situ* monitoring of the physiological activity, as well as structural organization of the organisms in the microenvironment.

Based on the green fluorescent protein (Gfp), we developed a new method [3] that specifically allowed us to monitor plasmid transfer in the environment. Applying 16S rRNA hybridization techniques and confocal laser scanning microscopy allowed exact mapping of the spatial distribution of transconjugants relative to the recipients and the total population in the microenvironment [4]. Lately, we have also developed monitor systems that allow for assessment of changes in the physiological activity of bacteria in biofilms [unpublished].

In the present work, quantitative data based on plating of cells collected from the effluent of flow chamber biofilms were compared with *in situ* studies of plasmid transfer and bacterial activity based on the detection of green fluorescent cells.

Materials and Methods

Strains and plasmids

A model biofilm community was cultivated as a mixture of three bacterial species, *Pseudomonas putida* RI (JB156, Nal^r), *Acinetobacter* spp. C6 (SMO112) and *Burkholderia cepecia* spp. D8 (SMO125). In the plasmid transfer studies, *P. putida* KT2442 modified by the insertion of the *lacI*^{Q} gene on the chromosome (SM1443), or *P. putida* RI (JB154, Rif^r), was used as donor. A modified version of the TOL plasmid (TOL*gfp*mut3b) was used as conjugative vehicle. The TOL-plasmid was modified by insertion of a cassette with a kanamycin gene, and the P*lac*$_{A1/O4/O3}$ promoter [2] fused to an enhanced version of the *gfp* gene (*gfp*mut3b) [5].

P. putida RI (SM1639) with a chromosomal insertion of a cassette carrying a kanamycin gene and the growth phase regulated rRNA promoter *rrnB* P1 fused to an unstable variant of the *gfp* gene, *gfp*(AAV) [1], was used for determination of changes in microbial activity in the biofilms.

Cultivation of strains and biofilms

Luria-Bertani broth (LB) was used for cultivation of overnight cultures and for plating experiments. When required, antibiotics were added to final concentrations of 50 µg/ml for rifampicin and nalidixic acid, and 10 µg/ml for kanamycin. The biofilms were cultivated as mixtures of the three model bacteria in rectangular two channel flow cells [8] (with dimensions of 1x4x40 mm) and supplied with a flow (flow rate = 0.2 mm/s) of FAB minimal media (1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl). Benzyl-alcohol at a concentration of 0.5 mM was used as the sole carbon source.

Simultaneous visualization of Gfp and rRNA

The probe PP986 (specific for *P. putida* subgroup A) labeled with CY3 fluorochrome was used for rRNA hybridizations. *In situ* detection of the biofilm cells expressing Gfp and simultaneous monitoring of the cells hybridized with the fluorochrome labeled rRNA specific probe, was performed by fixing and embedding of the biofilm followed by hybridization as described in Christensen et al. [4].

Microscopy and image analysis

All microscopic observation and image acquisition was performed on a Leica TCS4D confocal microscope (Leica Lasertechnik, GmbH, Heidelberg, Germany) equipped with 3 detectors and filter sets for simultaneous monitoring of FITC/GFP and CY3. Multichannel confocal images were processed using the IMARIS software package running on a Silicon Graphics computer and were prepared for display with Adobe Photoshop software.

Results and Discussion

A model biofilm community, consisting of the three species described above, was established by mixing overnight cultures of the strains and inoculating into flow chambers. Benzyl alcohol (the first degradation product after toluene via the TOL-pathway) was provided as the sole carbon and energy source for growth of the community. Of the three strains, the TOL-plasmid could only be established in *P. putida* RI, resulting in an approximately 10 to 20% increase in growth rate.

Quantitative data based on plating of cells collected from the flow channel effluent

A donor strain (KT2442, *lacI*^{Q}, rif^{r}, TOL*gfpmut*3A), which was not isogenic with the indigenous *P. putida* RI, was introduced two days after the initial colonization of the model community. This strain grew in batch culture with a doubling time similar to that of *P. putida* RI carrying the TOL-plasmid and, therefore, a growth advantage over the recipient cells in the model community was expected.

The incoming donor was introduced into separate flow chambers in three different concentrations (5 x 10^6, 5 x 10^7 or 5 x 10^8 cfu/ml of an overnight culture). Time course analysis of the population profile of cells collected from the effluents revealed that both

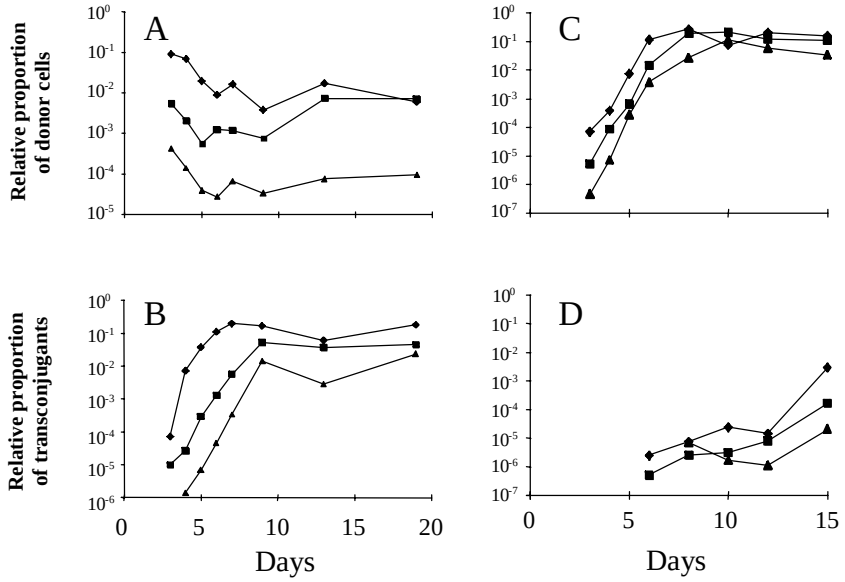

Figure 1. Time course analysis of the distribution of donor and transconjugant cells taken relative to the total number of cells collected from flow channel effluents. Donor was introduced at day 2 in three different concentrations: Panel A and B: 5 x 10^6 cfu/ml (▲), 5 x 10^7 cfu/ml (■) and 5 x 10^8 cfu/ml (◆) of donor (*P. putida* KT2442, *lacI*^{Q} TOL*gfp*mut3b) was introduced, and donor cells (Rif^{r}) (A) and transconjugants (Nal^{r}+ Km^{r}) (B) were enumerated. Panel C and D: 5·10^4 cfu/ml (▲), 5 x 10^5 cfu/ml (■) and 5 x ·10^6 cfu/ml (◆) of donor (*P. putida* RI, TOL*gfp*mut3b) was introduced, and donor cells (Rif^{r}) (C) and transconjugants (Nal^{r}+ Km^{r}) (D) were enumerated.

total cell counts (~10^7 cfu/ml) and the relative proportion of *P. putida* RI cells (~50% of total population) were not significantly affected by the introduction of the new strain.

Fig. 1a shows that the relative number of donor cells collected from the flow channel effluents the first few days after their introduction reflected the differences in the inoculation concentrations. Moreover, there was an initial phase of washing out of the strain. Thus, the non-isogenic nature of the *P. putida* KT2440 relative to the indigenous population seems to prevent this strain from establishing, despite its improved catabolic pathway encoded by the TOL-plasmid. In contrast, the fraction of transconjugants produced upon transfer of the TOL*gfp*mut3b plasmid to the indigenous *P. putida* RI cells increased rapidly until reaching a steady state level of approximately 10% (Fig. 1b). Based on this study alone we could not determine whether horizontal or vertical transfer caused the increase in number of transconjugants. Therefore, a separate experiment was performed where cells similar to the transconjugants from the above experiment (except for a change in the resistance marker from Nal^r to Rif^r) were introduced instead of *P. putida* KT2442. In this case, the introduced strain started to increase in numbers immediately (Fig. 1c) whereas conjugation to the indigenous recipient strain remained at relatively low levels (Fig. 1d). These observations suggest that the dominant mode of establishment of the TOL plasmid in the present community is through the growth advantage the plasmid confers to its host. Thus, despite the difficulty with which new organisms may be established in existing microbial communities, new genetic information can be introduced very effectively when located on mobile elements, even if the actual transfer rates are low.

Despite the rapid accumulation of *P. putida* RI cells carrying the TOL-plasmid, and their obvious growth advantage, the total counts in the effluent never exceeded more than approximately 50 % of the total number of *P. putida* RI cells in the community (plasmid carrier + plasmid free). Furthermore, although the transfer frequency between isogenic *P. putida* RI cells was nearly the same as between *P. putida* KT2442 and *P. putida* RI on agar plates (data not shown), the transfer frequency of the TOL-plasmid between isogenic *P. putida* RI cells in the biofilm was low. To understand the reason for this, methods for *in situ* monitoring of the structural organization of transconjugants relative to the potential recipient cells in the biofilm were applied.

Monitoring plasmid transfer and bacterial activity in biofilms

Tagging the TOL plasmid with a *gfp* gene (encoding the green fluorescent protein, GFPmut3b) fused to the strong *lac* promoter $P_{A1/O4/O3}$ (see Materials and Methods) allowed us to monitor the *in situ* establishment of the plasmid within the biofilm. In order to specifically follow the occurrence and growth of transconjugants we further inserted the *lacI* gene in the chromosome of the donor strain *P. putida* KT2442 resulting in repression of *gfp* expression from the plasmid. Thus, expression of the *gfp* gene will be induced only upon transfer of the TOL plasmid to a recipient in the community (*P. putida* RI) not harboring the *lacI* gene (zygotic induction of fluorescence).

The structural organization of the transconjugants relative to the indigenous recipient cells of *P. putida* RI was investigated 8 days after introduction of 10^8 cfu/ml donor cells of *P. putida* KT2442 carrying the plasmid TOL*gfp*mut3. The biofilm was fixed and embedded and *in situ* hybridization was performed in combination with SCLM.

The donor and recipient *Pseudomonas putida* strains cannot be distinguished with the specific 16S ribosomal DNA hybridization probe (PP986) used. However, disassembling of a number of steady state growing biofilms and plating of the biofilm cells on selective

plates showed that the number of the *P. putida* KT2442 donor cells were more than 50 times lower the number of *P. putida* RI recipient cells (data not shown). Therefore, most *P. putida* cells identified by the PP986 probe are assumed to be *P. putida* RI cells. Furthermore, the Gfp expressing cells of the same disassembled flow chamber biofilms were enumerated and found to correlate with the number of kanamycin and nalidixic-acid resistant cells (transconjugants) determined by plating on the selective plates, strongly indicating that all the transconjugants in the biofilm would also appear as green fluorescent. In other words, both oxygen tension and biosynthetic levels were high enough for maturation and expression of Gfp protein to produce fluorescent cells in the entire biofilm.

Fig. 2 shows horizontal and vertical cross sections of a representative region of the biofilm. As shown in Fig. 2c, with a few exceptions the *P. putida* RI micro-colonies were all covered with green-fluorescent transconjugant cells on top. Although the transconjugant and recipient cells seemed to be extremely tightly associated in the micro-colonies, horizontal transfer of the TOL plasmid through the entire recipient colony was not observed. A number of explanations may account for these observations, but one of the most obvious is that TOL plasmid transfer is strongly dependent on the substrate concentrations, i.e. the bacterial growth rate [6, 7].

Therefore, to further investigate a possible relationship between the physiological activity of *P. putida* RI and the lack of efficient plasmid transfer into the center of micro-colonies, a new biofilm experiment was performed. In this experiment the recipient strain

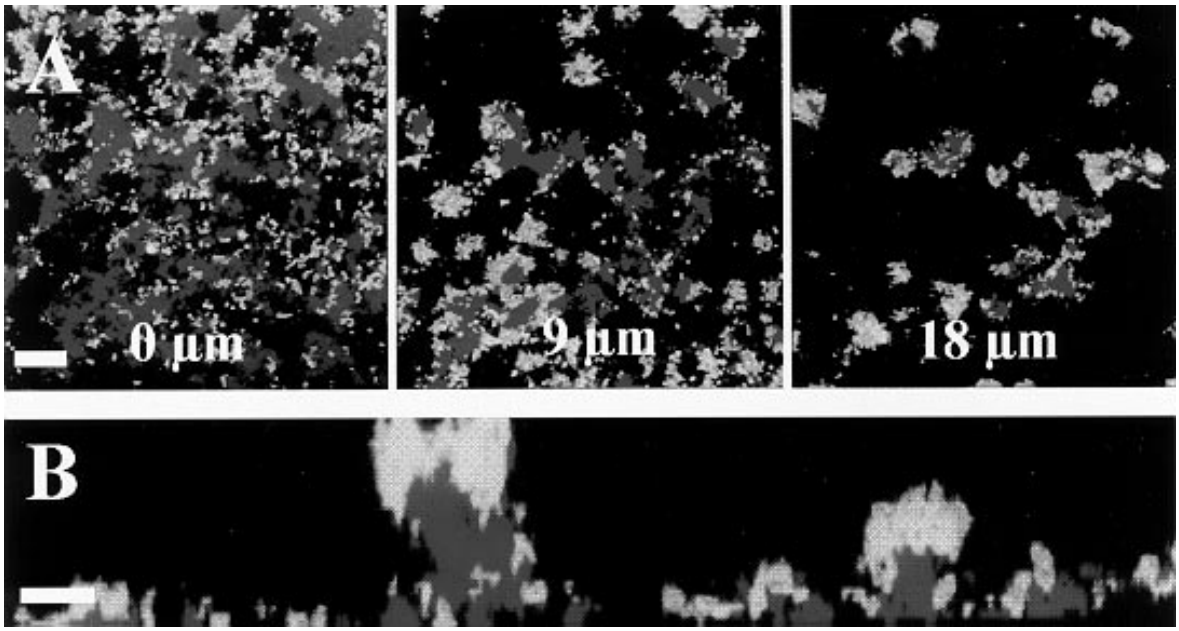

Figure 2. Horizontal and vertical cross sections through a representative region of the biofilm illustrating the spatial distribution of green fluorescent transconjugants (green/yellow) relative to the non infected *P. putida* cells (red). The organisms *P. putida* (red) were identified by hybridization. After hybridization, green fluorescent transconjugants appear as either yellow or green depending on the ratio between the intensity of the green GFP signal and the red hybridization signal. Shown are horizontal cross sections (A) from three different depths of the biofilms and (B) a single vertical cross section from the same region of the biofilm. Bars indicate 20 µm in panel A and 10µm in panel B.

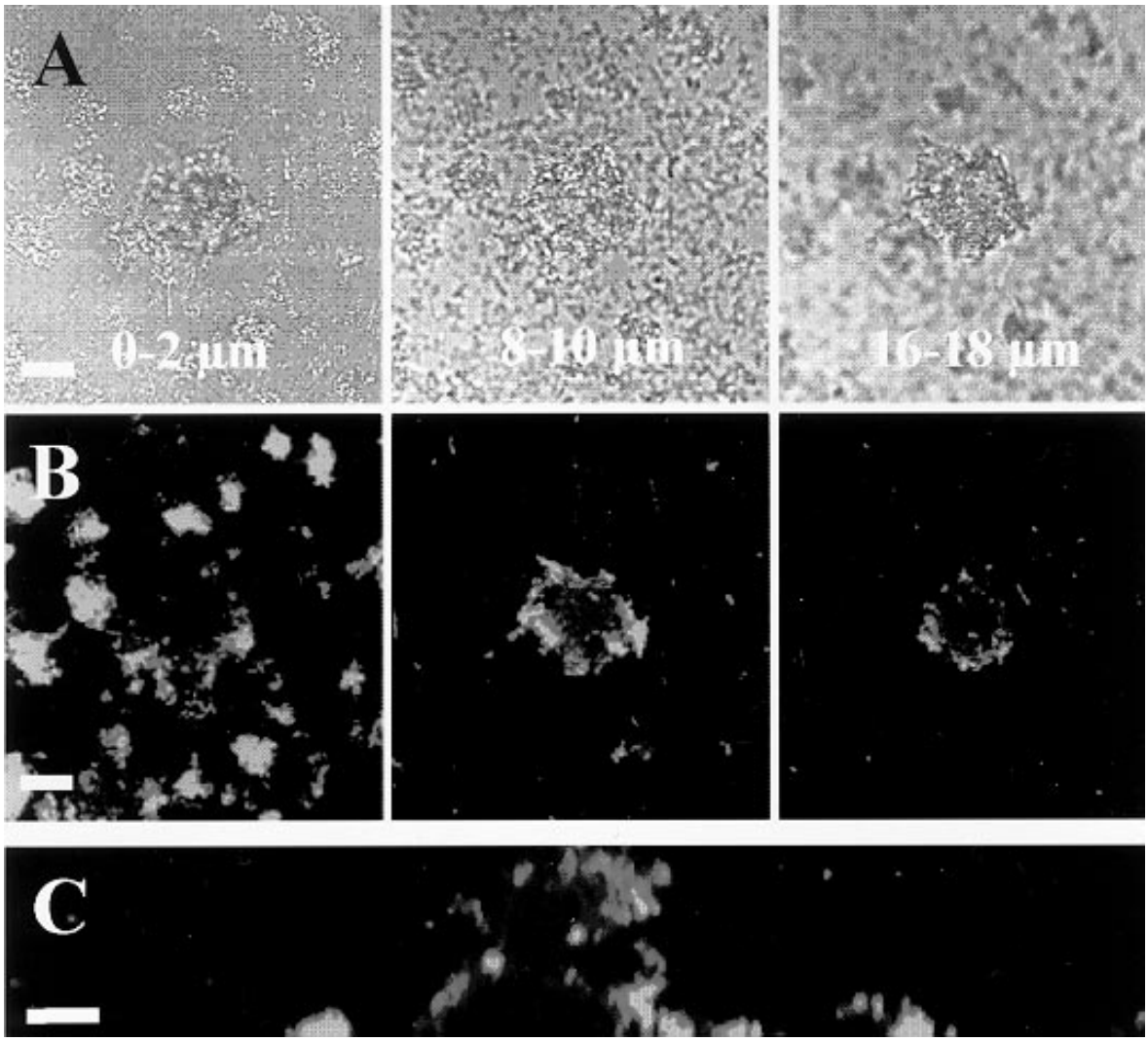

Figure 3. Illustration of the spatial distribution of actively growing cells (the green fluorescent areas) in a biofilm grown for one day. Panel A and B are horizontal cross sections captured in different depths of the same viewing field. Bright field microscopy (A) was used to visualize the total number of cells in the biofilm and (B) SCLM was used to monitor the green fluorescent signal emitted from the actively growing *P. putida* RI cells. Panel C shows a vertical cross section of the same region of the biofilm. Note that void regions below the green fluorescent areas in the vertical cross section are filled with bacteria with a low fluorescent intensity (low growth activity). These cannot be monitored by the SCLM since it requires the observed objects to fluoresce. Bars indicate 20 µm in panel A and B, and 10 µm in panel C.

P. putida RI (JB156, Nal^r) was replaced with a *P. putida* RI carrying a cassette with the growth phase regulated ribosomal promoter *rrnB* P1 fused to the *gfp* gene *gfp*(AAV) encoding an unstable variant of the Gfp protein [1]. Due to instability of the Gfp protein is it possible to determine changes in the growth physiology of the biofilms grown *P. putida* RI cells? The physiological activity of bacteria was analyzed by direct microscopic inspection one day after initial colonization of the biofilm organisms. As shown in Fig. 3b and 3c distinct areas with high cell activity appear (visualized as bright green fluorescent cells), which is dominant near the surface/edge of the micro colonies. The pattern observed is strikingly similar to the pattern observed in Fig. 2 for the establishment of

transconjugants. The correlation between activity and transfer may suggest that there is a nutrient diffusion gradient through a micro-colony allowing growth of only the surface cells, and that plasmid transfer only occurs between these actively growing cells.

Conclusion

These studies have indicated that the structure/function relationships with respect to distribution of growth activity in the biofilm, together with the selective advantage offered by the introduction of a new biochemical pathway, are more important than the conjugative properties of the plasmids in determining the rate of dispersal of plasmids through the biofilm community. It will be interesting in the future to see if this pattern of gene transfer also exists in other biofilm scenarios and with other plasmids.

Acknowledgments

This work was supported by grants from the Danish Biotechnology Program. We also thank Anne Nielsen and Tove Johansen for expert technical assistance.

References

1. Andersen JB, Sternberg C, Poulsen LK, Petersen S., Givskov M and Molin S.1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64:2240-2246.
2. Bujard H and Lanzer M. 1988. Promoters largely determine the efficiency of repressor action. Proc Natl Acad Sci USA. 85:8973-8977.
3. Christensen BB, Sternberg C, and Molin S. 1996. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (Gfp) from *Aequorea victoria* as a marker. Gene 173:59-65.
4. Christensen BB, Sternberg C, Andersen JB, Eberl L, Møller S, Givskov M and Molin S. 1998. Establishment of new genetic traits in a microbial biofilm community. Appl Environ Microbiol 64:2247-2255.
5. Cormack BP, Valdivia RH, and Falkow S. 1996. FACS-optimized mutants of the green fluorescent (GFP). Gene 173:33-38.
6. Smets BF, Rittmann BE, and Stahl DA. 1993. The specific growth rate of *Pseudomonas putida* PAW1 influences the conjugal transfer rate of the TOL plasmid. Appl Environ Microbiol 59:3430-3437.
7. Smets BF, Rittmann BE, and Stahl DA. 1995. Quantification of the effect of substrate concentration on the conjugal transfer rate of the TOL plasmid in short-term batch mating experiments. Letters in Appl Microbiol 21:167-172.
8. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell SJ, and Caldwell DE. 1994. Multicellular Organization in a Degradative Biofilm Community. Appl Environ Microbiol 60:434-446.